REMARKS

The Claim Amendments

Applicants have cancelled claim 4 and have amended claims 1 5-8, and 22, without prejudice.

Claim 1, as amended, recites a method for the selection and generation of a transgenic *Linum usitatissimum* plant. This amendment does not change the scope of the claim. It merely clarifies the claim.

Applicants have additionally amended claims 1 and 22 to incorporate into them the limitations of claim 4. Applicants have accordingly cancelled claim 4 and have amended claims 5-8 to correct their dependency.

These claim amendments are supported throughout the specification and by the claims as originally filed. Therefore, the claim amendments introduce no new matter.

Applicants expressly reserve the right to pursue the subject matter of the claims, as originally filed, in future applications that claim benefit or priority from the instant application.

The Rejections

35 U.S.C. § 112: Enablement

(a) Alleged Unpredictability

The Examiner has maintained the rejection of claims 1 and 4-23 as allegedly lacking enablement for the full scope of the claimed subject matter. The Examiner asserts that there is "evidence of unpredictability at each step of the claimed method" (Office Action, page 2). In particular, the Examiner maintains that the steps of introducing foreign DNA into flax, as recited in part (a) of claim 1, and of recovering whole

transgenic flax plants, as recited in part (e) of claim 1, are unpredictable. Applicants traverse.

In their November 8, 2006 Response, Applicants explained that the step of introducing a recombinant DNA molecule into a flax cell is "relatively easy in most dicotyledonous species attempted, through the use of either Agrobacterium vectors, electroporation, or other methods of DNA delivery into cells" (November 8, 2006 Response, page 7, citing McHughen and Jordan, 1989 Plant Cell Reports (7): 611-614, ("McHughen and Jordan")). Applicants also stated that the technical problem underlying this invention relates to the selection of stably transformed plants of the genus *Linum*. This problem is solved by the disclosure and the teachings of this application.

The Examiner, however, contends that Applicants' arguments are not commensurate in scope with the claims because "none of the claims presently being examined recite the 'selection of stably transformed plants'" (Final Office Action, page 3). The Examiner also maintains, in spite of McHughen and Jordan, that the step of introducing foreign DNA into flax is unpredictable. Finally, the Examiner argues that part (e) of claim 1, which recites "regenerating a transgenic *Linum usitatissimum* plant from said transgenic callus", is unpredictable based on the prior art. The Examiner states, "The prior art cited by the Examiner demonstrates the difficulty in obtaining high frequencies of, and/or morphologically normal, flax transformants. The cited prior art also discusses genome dependence and explant type as contributing factors to said unpredictability, in addition to antibiotic type" (Final Office Action, paragraph bridging pages 3 and 4). Applicants traverse.

(1) The Literal Words of the Claims

While the claims did not literally recite the "selection of stably transformed plants", the preamble of claim 1, which recited "generation of a transgenic *Linum usitatissimum* plant", accurately captured the technical problem. Indeed, the term "generation" encompasses "selection". However, solely to expedite prosecution, Applicants have amended claim 1 to recite "selection" in addition to "generation". This amendment does not alter the scope of the claimed invention. It clarifies the claim's original meaning and scope and should overcome the Examiner's concern about Applicants' arguments and the scope of the claims.

(2) Step (a): Introducing DNA into a Flax Cell

The step of introducing DNA into a flax cell is not unpredictable. Applicants reiterate their arguments of record, where they provided McHughen and Jordan as evidence that this step is enabled.

Ling and Binding, 1997 *J. Plant Physiol.* 151: 479-488 ("Ling and Binding", enclosed as Exhibit 1) likewise describes protoplast transformation of *Linum suffruticosum* and plastocyte transformation of *Linum usitatissimum*. In this report, isolated plastocytes of *L. usitatissimum* "showed a relatively high transformation competence" (page 486, last paragraph of second column). This confirms that the step of introducing DNA into isolated plastocytes was efficient and effective using this single-cell transformation system.

Wijayanto and McHughen, Genetic transformation of Linum by particle bombardment, 1999 *Congress on in Vitro Biology*, June 5-9, 1999 ("Wijayanto and McHughen", abstract enclosed as Exhibit 2) also documents that step (a) is not unpredictable. Wijayanto and McHughen describes particle bombardment of *L. usitatissimum*

cells as a method of transforming flax. See discussions below under **Particle bombardment**.

Accordingly, as evidenced by McHughen and Jordan and the additional documents cited above, the art provides several different methods for introducing DNA into flax cells. This step of the claimed invention is, therefore, fully enabled across the entire claimed scope.

(3) Step (e): Regenerating a Transgenic Plant

The step of regenerating a transgenic plant is enabled.

In the April 24, 2006 Office Action (pages 4-5), the Examiner pointed to certain recitations of the prior art as supposed support for her view that this step is unpredictable:

> Zhan et al., 1988 ("Zhan") examined *Agrobacterium rhizogenes*-infected flax cotyledons, which produced transgenic plantlets with root systems characterized by plagiotropic behavior.
>
> McHughen and Jordan used *Agrobacterium tumefaciens* to infect flax hypocotyls and selection on kanamycin, showing that escape regenerants may contain stable transgenic cells which may be rescued using a second cycle of regeneration or by testing a larger number of progeny.
>
> Bretagne-Sagnard et al. 1996 ("Bretagne-Sagnard") recite that spectinomycin is more suitable for genetic engineering of flax than animoglycoside resistance.
>
> Ling, 1997 ("Ling"), recites a protoplast transformation system that produced plants, some of which exhibited morphological abnormalities.

These recitations do not show that regeneration of transformed plants using the methods of the claimed invention is unpredictable. For example, Zhan states that "in at least two cases, some side shoots recovered normal morphology during

subculture of transformed plants" (page 556, second column). Zhan also states on page 557

> To the best of our knowledge this is the first report of the regeneration of flax transformed by *A. rhizogenes*. These results show that the transformation by *A. rhizogenes* is an **effective alternative** to transformation by disarmed strains of *A. tumefaciens* for the genetic engineering of plants, especially when it is not possible to regenerate shoots readily from callus but is possible from roots. (**emphasis added**)

Accordingly, Zhan characterizes the transformation method as an "effective", not unpredictable, means of obtaining genetically engineered plants.

McHughen and Jordan also fails to provide evidence of unpredictability. McHughen and Jordan does not characterize the transgenic plants as having any abnormalities. McHughen and Jordan teaches that "transgenic segregants show **normal** green leaves and **normal** root growth" (page 612, first column, **emphasis added**).

Bretagne-Sagnard states in the Introduction, following a summary of the state of the art, that "there remains a need for a faster, more efficient and reliable transformation and regeneration system for the introduction of foreign genes into flax" (which the claimed invention achieves). Bretagne-Sagnard concludes from its study that "spectinomycin resistance is more suitable for genetic engineering of flax than aminoglycoside resistance". This conclusion does not provide any evidence that the success of the claimed method of the instant application is unpredictable. Rather, the conclusion addresses only the efficiency of different antibiotics in a method that employs *only one* antibiotic.

Finally, Ling recites a transformation system that produced plants, only some of which exhibited morphological abnormalities, which indicates that the remaining transformed plants were morphologically normal.

Accordingly, the cited documents demonstrate that skilled artisans were, at the application's priority date, generating morphologically normal transformed flax. The cited documents therefore fail to show that the regeneration of transformed flax was not enabled at the time of the application's priority date.

Other art (McHughen and Holm 1991 *Euphytica* 55: 49-56, abstract enclosed as Exhibit 3, "McHughen and Holm") also confirms that this step is not unpredictable. McHughen and Holm examines two transformed linseed flax lines and shows that "there were no significant differences between the transgenic lines and the parent for any agronomic trait measured in untreated soil, indicating that there is no detrimental effect of T-DNA or foreign gene expression". This study therefore provides further evidence that skilled artisans were generating agronomically normal transformed flax prior to the instant application's priority date.

(4) The Claimed Invention

The claimed invention is patentable because it provides a novel, more efficient and more reliable method for generating transgenic flax. Indeed, as noted above, Bretagne-Sagnard states in the Introduction, "there remains a need for a faster, more efficient and reliable transformation and regeneration system for the introduction of foreign genes into flax". The claimed invention meets this need by providing novel methods employing two different antibiotics for the selection and generation of transgenic

flax. The specification fully enables the claimed invention and demonstrates by working examples the success of the claimed methods.

Accordingly, even if the regeneration of a transgenic plant were unpredictable in the prior art (which it was not), Applicants' claims would still be patentable. These claims, unlike the prior art relied on by the Examiner, employ two different antibiotics in the selection and regeneration of a transgenic plant. The cited documents therefore cannot, and do not, provide any evidence of unpredictability. For example, regardless of whether or not the methods in the cited documents generate morphologically abnormal transgenic plants, the claimed method was shown to generate, for three different flax varieties, transformed rooted plants that are capable of flowering, producing seed, and that are "morphologically similar to their wild-type control variety" (page 5 of the specification, lines 2-4). Applicants have, therefore, provided working examples demonstrating that the claimed methods result in the generation of morphologically normal transgenic flax. The claimed invention is, thus, enabled throughout its scope.

(b) The antibiotics

The Examiner has also maintained that claims 1 and 4-23 do not contain all of the essential elements of the invention. These essential elements are, according to the Examiner, the use of *Agrobacterium* for the step of introducing DNA into a flax cell and one of the specific antibiotics recited in claim 4. Applicants traverse.

The claimed invention is enabled for the full scope of claim 1 and should not be restricted to the specific antibiotics

recited in claim 4. The Examiner states, "Applicant's specification merely asserts that hygromycin may work, without supplying any data to prove this assertion" (Final Office Action, paragraph bridging pages 4-5). Yet, use of hygromycin to select transformed flax cells was known in the art. See Ling and Binding, for example, which describes the selection of hygromycin B-resistant calli following protoplast transformation of *Linum suffruticosum.* The claimed invention is, therefore, enabled for the antibiotics recited in claim 4 as well as for antibiotics that were used and known in the art as of the priority date.

Nonetheless, and solely to expedite prosecution, Applicants have amended claim 1 to include the specific antibiotics of claim 4. Applicants' amendment is made without prejudice to Applicants' rights to pursue the subject matter of previously pending claim 1 in future applications.

(c) Particle Bombardment

The disclosure of particle bombardment both in the instant application and in the prior art fully enables the method of particle bombardment for introducing DNA into *Linum usitatissimum* cells. This method, therefore, does not involve undue experimentation.

The Examiner argues that US patent 5,973,227 ("the '227 patent") presents "numerous modifications and requirements for successful microprojectile transformation of flax" such as "use of 'flax hypocotyl tissue comprising epidermal tissue' (claim 1) and preculturing the hypocotyl tissue for a period of 4 days on a medium consisting of 3% sucrose, 0.8% agar, 1.0 mg/i/L BA and 0.02 mg/L NAA (column 4, lines 45-53)" (Final Office Action, pages 5-6). This is irrelevant. The instant application teaches transfection of hypocotyls comprising epidermal tissue

(see page 4, line 2, for example). In addition, the '227 patent does not teach that the other supposed "modifications" or "requirements" are necessary to practice particle bombardment of flax. Indeed, claim 1 of the '227 patent does not recite the specific medium or preculturing period cited by the Examiner. Claim 1 of US 5,973,227 recites

> 1. A method for producing a transgenic flax plant, comprising:
> preculturing a flax hypocotyl tissue comprising epidermal tissue on a regeneration induction medium;
> bombarding the precultured flax hypocotyl tissue with a microprojectile that comprises a nucleic acid, thereby producing a transformed flax hypocotyl comprising transformed flax cells that comprise the nucleic acid; and
> regenerating a transgenic flax plant from the transformed flax hypocotyl tissue.

See also Wijayanto and McHughen (Exhibit 2). Wijayanto and McHughen examined different factors that influence DNA delivery by particle bombardment in flax and published their results before the priority date of the instant application. Accordingly, the instant application, in combination with the state of the art on particle bombardment as well as other techniques of introducing DNA into plant cells, enables the skilled artisan to practice the full scope of the claimed invention without undue experimentation.

CONCLUSION

For at least the reasons presented above and the claim amendments, Applicants request that the Examiner reconsider and allow the pending claims.

The Examiner may address any questions raised by this submission to the undersigned at 212-596-9000.

In the accompanying Transmittal Form, Applicants have authorized that any fee required be charged to Deposit Account No. 06-1075 under order number 003747-0068.

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Respectfully submitted,

By _____
James F. Haley, Jr.
Reg. No. 27,794

Fish & Neave Intellectual
Property Group
ROPES & GRAY LLP
Customer No. 1473
1211 Avenue of the Americas
New York, New York 10036
Tel.: (212) 596-9000
Fax: (212) 596-9090
Attorney For Applicants

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Transformation in protoplast cultures of *Linum usitatissimum* and *L. suffruticosum* mediated with PEG and with *Agrobacterium tumefaciens*

HONG-QING LING[1]* and HORST BINDING[2]

[1] Institute for Plant Genetic and Crop Plant Research, Corrensstraße 3, D-06466 Gatersleben, Germany

[2] Botanical Institute of the Christian-Albrechts-University, Olshausenstraße 40, D-24098 Kiel, Germany

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Summary

Transgenic plants of *Linum usitatissimum* L. and *L. suffruticosum* ssp. *salsoloides* (Lam.) Rouy. were obtained through direct transformation of protoplasts with PEG, as well as through *Agrobacterium tumefaciens*-mediated transformation of plastocytes. Direct transformation with the marker gene *HPT* in *L. suffruticosum* occurred in relative frequencies of about 2×10^{-3}. The efficiencies of *Agrobacterium*-mediated transformation of plastocytes with the genes *NPT II* and *GUS* strongly depended on the plant genotypes and on the bacteria densities as concluded from experiments with six genotypes of *L. usitatissimum*. Abnormal flower morphology observed in two transgenic lines of the cultivar Bionaa was transmitted to the progeny. The efficiencies and advantages of the techniques used here are compared with the previously successfull *Agrobacterium*-mediated hypocotyl transformation.

Key words: Linum, transformation, Agrobacterium tumefaciens, PEG, protoplasts.

Abbreviations: BAP = 6-benzylamino purine; *GUS* = β-glucuronidase gene; *HPT* = hygromycin phosphotransferase gene; *NOS* = nopaline synthase gene; *NPT II* = neomycin phosphotransferase II gene; 35S = 35S promotor of the cauliflower mosaic virus.

Introduction

Genetic engeneering in plants contributes to basic research and plant breeding. It serves in understanding molecular regulation processes in development, the exploration and utilization of pathogen defense mechanisms, and in raising the quality and quantity of plant products. Since the first transgenic plants were regenerated in tobacco (Horsch et al., 1984; Block et al., 1984), several transformation techniques were developed (s. review, Potrykus, 1991).

Transgenic plants of *Linum usitatissimum* had ealier been obtained from hypocotyl and cotyledon explants, which were primarily cocultured with *Agrobacterium tumefaciens* or *A. rhizogenes* containing disarmed vectors (McHughen et al., 1986; Basiran et al., 1987; Jordan and McHughen, 1988; Zhan et al., 1988; McHughen, 1989; Dong and McHughen, 1991). Transformation efficiency was generally very low (McHughen et al., 1989). Moreover, transgenic calli formed from the inoculated tissues (hypocotyl and cotyledon) were a complex mixture of non-transformed and several types of independently transformed cells, and chimeric shoots were often regenerated (McHughen and Jordan, 1989). These made it more difficult to acquire stabile transgenic plants.

Transformation with protoplasts was described as an effective alternative (Potrykus, 1991). In *Linum*, protoplast transformation has not yet been reported. The aims of the experiments presented here were the evaluation of different protoplast transformation techniques for *Linum* and the introduction of differentially selective markers into species for studies on their somatic compatibility (Ling, 1992).

Regeneration from isolated protoplasts of *Linum* species was reported (Binding et al., 1982; Barakat and Cocking,

* Correspondence.

1983; 1985; Ling and Binding, 1987; 1992; Binding et al., 1992; Zhan et al., 1989). While regeneration capability in *L. usitatissimum* was rather low, the protoplasts of *L. suffruticosum* showed a particularly high regeneration frequency (Ling and Binding, 1987).

Materials and Methods

Plant material

From *Linum suffruticosum* ssp. *salsoloides* (Lam.) Rouy. an established shoot culture of a wild biotype (Ling and Binding, 1987) was used. Seeds of six genotypes of *Linum usitatissimum* L. (Bionaa, C.A.N. 2612-A, IR 00610, Langeland B, Otofte 15/47 and Ottawa) were kindly provided by M. Dambroth (Institute for Plant Breeding of FAL Braunschweig, Germany). They were surface sterilized with a solution of 5 % NaClO for 10 min and washed twice with sterile distilled water. The seeds were germinated on MS medium (Murashige and Skoog, 1962) with 2.5 µmol/L BAP at 25 ± 2 °C and a 16 h day by white fluorescent light at about 30 µmol $m^{-2} s^{-1}$.

Protoplast isolation and culture

Cotyledons 7–10 days after germination of *L. usitatissimum* and about 5 mm long upper parts of shoots of *L. suffruticosum* were harvested and cut into small segments with a sterile razor blade. Slices (1 g each) were incubated in a 25 mL solution containing 3 % w/v Rohament PC enzyme (Röhm, Darmstadt, Germany), 5 mmol/L $Ca(NO_3)_2$, and 0.5 mol/L sorbitol in a 200 mL flask on a roller (20 rpm). After 14–16 h, the suspensions were poured through nylon sieves with pore size 100 µm and centrifuged at about 100 g_n for 5 min. The protoplasts were purified by floating with 0.6 mol/L sucrose solution at about 80 g_n for 10 min. An additional centrifugation followed in washing solution V47/NaCl (one part solution of V-KM salts and nine parts 0.3 mol/L NaCl; Binding and Nehls, 1977) at about 80 g_n for 5 min. Protoplast culture was performed as described by Ling and Binding (1987). Modifications due to the demands of transformation are mentioned in the respective sections.

Vectors and plasmids

The plasmid pGL2 (Karesch et al., 1991), used for direct transformation, was kindly provided by F. Köhler, Institute for Applied Genetics of the Freie Universität, Germany, with the consent of the constructor J. Paszkowski (ETH, Zürich, Switzerland). The plasmid pGL2 was derived from plasmid pDH51; the 1033 bp sequence coding for the hygromycin phosphotransferase (*HPT*) is inserted into the polylinker sequence at the BamH I position. The plasmids were cloned in strain JM 83 of *Escherichia coli* and isolated with the Qiagen technique (Diagen, Düsseldorf, Germany).

The *Agrobacterium tumefaciens* strains GV2260 (Deblaere et al., 1985) and C58C1Rif®, which contained the non-oncogenic binary vector pBin19 35S*GUS* (Vancanneyt et al., 1990) and the cointegrative vector pGV3850*HPT* (Baker et al., 1987), respectively, were provided by L. Willmitzer (Institute for Gene Biology Research, Berlin, Germany). The vector pBin19 35S*GUS* carries the reporter gene GUS (the β-glucuronidase gene under the control of the 35S promotor of the cauliflower mosaic virus) and the marker gene *NPT II* under the control of *NOS* promotor in its T-DNA, whereas the vector pGV3850*HPT* has only the marker gene *HPT* with the *NOS* promotor. For transformation, bacteria were grown in Luria broth medium with 50 mg/L kanamycin sulfate for strain GV2260 pBin 19 35S*GUS* and with 100 mg/L spectinomycin and 100 mg/L rifampicin for strain C58C1Rif® pGV3850*HPT* at 28 °C overnight with swirling (200 rpm).

Transformation and growth of transformants

A) Direct protoplast transformation

Direct transformation was achieved with *L. suffruticosum* because of its good regeneration capability from isolated protoplasts. A protocol modified from Negrutiu et al. (1987) was followed. Protoplasts were suspended in W5 solution at a density of 1.0×10^6 mL and distributed in 0.2 mL aliquots each into a plastic Petri dish of 60 mm diameter. Subsequently, 20–50 µg circular pGL2-DNA and 50 µg carrier DNA (calf thymus, Sigma) were added. The suspension was mixed with 100 µL PEG-CMS solution (30 % of PEG 6000, Firma Merck). After incubation for 30 min, the transformation mixture was stepwise diluted with 10 volumes of V47/NaCl and then centrifuged for 5 min at 80 g_n. Control protoplasts were treated in the same way but without the plasmid DNA. After the transformation treatment, $0.5–1.0 \times 10^6$ protoplasts were suspended in 1 mL liquid V-KM medium and cultured for 2–4 days. Then, the cells were embedded in a bottom gel layer through replacement of 0.5 mL supernatant with 0.5 mL V-KM medium containing 2 % agarose (type VII, Sigma), which was heat-liquefied and cooled to 40 °C. The gel layer was covered with 1 mL of liquid V-KM medium, which was exchanged every 2 to 3 days. Ten days after the transformation treatment, the liquid was replaced with selection medium (V-KM medium with 30 mg/L hygromycin B), which was renewed every 3–4 days. Resistant calli were transferred onto B5C medium (B5 medium, Gamborg et al., 1968, supplemented with 50 mL/L coconut endosperm and 2.5 µmol/L BAP) with 30 mg/L hygromycin B to regenerate shoots. Shoots were subcultured on MS medium with 2.5 µmol/L BAP and 30–40 mg/L hygromycin B.

B) Plastocyte transformation

Protoplasts were precultured in liquid V-KM medium at titres of $10^5–10^6$/mL for 2–4 days to form plastocytes, and part of them already divided. Agrobacteria were cultured overnight up to a concentration of 10^9 bacteria/mL. Each 1 mL of the plastocyte suspension was cocultured with 100 µL or 10 µL of the bacteria suspension, *L. usitatissimum* with the strain GV2260 pBin19 35S*GUS* and *L. suffruticosum* with the strain C58C1Rif® pGV3850*HPT*. After 16–24 h, the cells were embedded in agarose, as described in the section of direct transformation, and covered with 1 mL V-KM medium containing 500 mg/L claforan in order to eliminate the bacteria. The medium was exchanged every 2–3 days. At the 10th day after inoculation with bacteria, the media were replaced with selection medium (V-KM medium containing 500 mg/L claforan and 50 mg/L kanamycin sulfate for *L. usitatissimum* or 30 mg/L hygromycin B for *L. suffruticosum*). Four to five weeks later, the microcalli formed were transferred onto B5C medium for *L. usitatissimum* and B5C medium supplementing 5.0 µmol/L NAA and 0.5 µmol/L 2,4-D for *L. suffruticosum*, and cultured for 2–3 weeks. To induce organogenesis, calli of *L. usitatissimum* were placed onto MS medium containing 2.5 µmol/L BAP and 100 mg/L kanamycin sulfate and calli of *L. suffruticosum* onto B5C medium with 30 mg/L hygromycin B. Calli and regenerated shoots were subcultured every 4 weeks.

C) Rooting and growth in soil

The regenerated shoots were rooted on phytohormone-free MS medium with 100 mg/L kanamycin sulfate for *L. usitatissimum* and with 5.0 µmol/L NAA, but without the selective antibiotic for *L. suffruticosum*. The rooted shoots of *L. usitatissimum* were planted in soil and grown in the growth chamber to maturity.

Histochemical assay of GUS gene activity

Sections of regenerated calli and shoots of the transformants and the control of *L. usitatissimum* were stained with a solution of 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc; Jefferson, 1987) at 37 °C for 14–20 h.

Molecular biological analysis

Total DNA of transgenic calli or shoots was isolated following the protocol of Draper and Scott (1988). About 10 µg DNA were digested with the restriction enzymes Hind III, EcoR I or BamH I at 37 °C for 16–20 h. The DNA fragments were separated on 0.8 % agarose gels and blotted on positively charged nylon membranes (Pall, USA) through the alkaline capillar transfer technique (0.4 mol/L NaOH). Then, the DNA was fixed on the membranes at 80 °C for 15 min.

Probes for the detection of the introduced genes were prepared in the following way: The 1033 bp *HPT* gene was cut off from the plasmid pGL2 with BamH I, an about 500 bp sequence of the 35S promotor from the plasmid pGL2 with EcoR I/BamH I, the 800 bp *NPT II* gene from the plasmid pHP23 (Paszkowski et al., 1988) with Hind III and the about 2.5 kb *GUS* gene from the plasmid Bin19 35S*GUS* with Hind III, respectively. Amounts of 10–50 ng DNA of each probe were labelled with (α-^{32}P)dATP as described by Feinberg and Vogelstein (1983). The hybridization to the DNA on

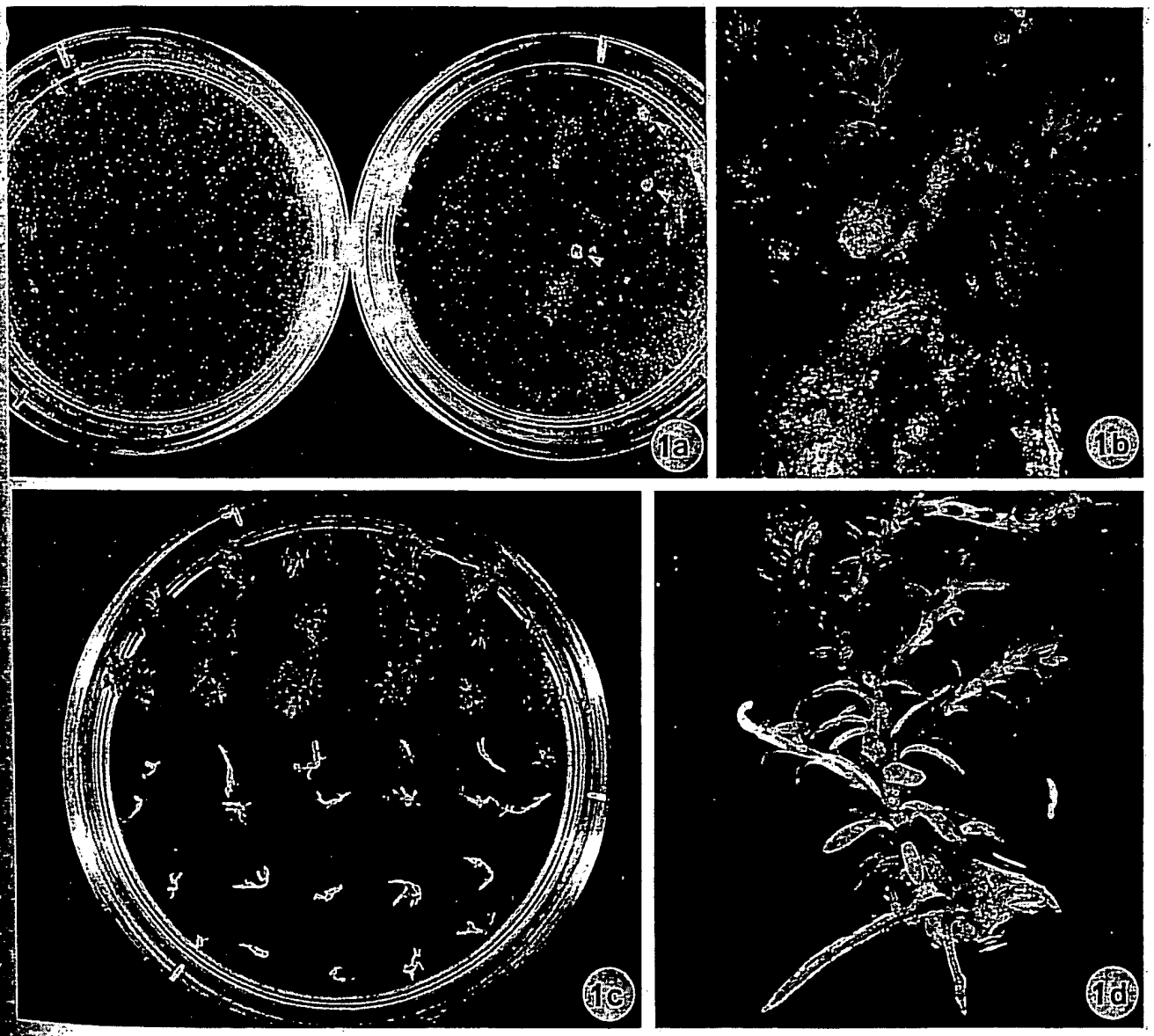

Fig. 1: Direct transformation of protoplasts in *L. suffruticosum* with the plasmid pGL2: 1 a) resistant calli (right, s. arrow heads) formed in V-KM medium with 30 mg/L hygromycin B, 30 days after the transformation treatment, and control (left); 1 b) resistant calli with shoots formed on B5C medium with 30 mg/L hygromycin B; 1 c) transgenic shoots (upper part) showing resistance against hygromycin B; the wild type shoots (lower part) died off within 10 days on MS medium with 2.5 µmol/L BAP and 30 mg/L hygromycin B; 1 d) transgenic plantlet, rooted on MS medium containing 5 µmol/L NAA.

the nylon membranes was achieved by following the protocol of Amersham (Braunschweig, Germany). The membranes were washed and coated with Kodak X-film for autoradiography.

Results

Protoplast transformation in Linum suffruticosum

High degrees of protoplasts were destroyed by the PEG, the others became fragile. Therefore, recovery culture of the protoplasts in liquid for 2–4 days was necessary before embedding in agarose. No differences were observed during the recovery culture in the continuous dim light or in darkness. Anyhow, the frequencies of callus formation in transformation experiments were significantly reduced as compared with the control (Table 1).

Hygromycin B-resistant calli grew in the selection medium within 4–5 weeks (Fig. 1 a) up to about 2 mm in diameter. They were transferred onto organogenesis medium B5C with 30 mg/L hygromycin B. All of the calli regenerated adventitious shoots on this medium (Fig. 1 b), which became visible from the 50th day. The transgenic shoots grew vigorously (Fig. 1 c), whereas control wild type shoots died within 10 days after their transfer onto the selective medium. Roots were formed on the non-selective MS medium with NAA (Fig. 1 d).

The absolute transformation frequencies (numbers of resistant calli/numbers of protoplasts) ranged between 1.7×10^{-6} and 3.3×10^{-6} (Table 2), the average being 2.2×10^{-6}. The relative transformation rates as compared with the control protoplast-to-callus efficiences (s. Table 1) varied from 1.7×10^{-3} to 3.3×10^{-3}.

For molecular analysis of the transgenic lines, total DNA was isolated from shoots of eleven lines and the wild type. The DNA was digested with the restriction enzymes BamH I and Hind III, respectively. Autoradiograms of the Southern hybridization with the *HPT* probe showed that all of the eleven transgenic lines contained the *HPT* sequence, whereas no positive signal was obtained with a corresponding wild type DNA preparation (Fig. 2). Most of the lines possessed the complete 1033 bp *HPT* sequence, which could be cut out with BamH I. Also the blot of line Td18-2-1 showed the hybridization signal, but no 1033 bp long fragment could be excised with BamH I; although there is no digestion position for Hind III within the *HPT* sequence, a short *HPT*-positive fragment (about 300 bp) appeared by digestion with this enzyme (s. Fig. 2). The shoots of this line grew as well as the other lines on the selection medium.

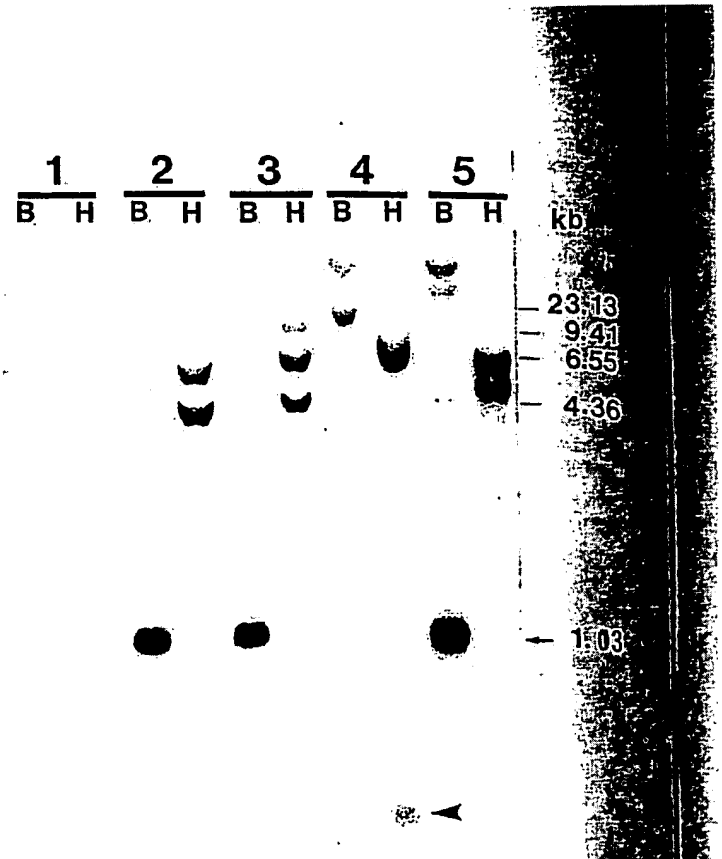

Fig. 2: Molecular analysis of transgenic lines of *L. suffruticosum* through Southern hybridisation. Total DNA was digested with BamH I (B) and Hind III (H), respectively, fractionated by agarose gel electrophoresis and probed with the 1033 bp *HPT*-gene sequence labelled with ^{32}P. No.1: wild type DNA as a negative control; Nos. 2–5: transgenic lines. The thin arrow shows the 1033 bp *HPT* fragments, which were cut off with BamH I. Line Td18-2-1 (No. 4) showed a probe-positive fragment of about 300 bp cut with Hind III (s. thick arrow head).

Table 1: Effect of the transformation treatment on callus formation from protoplasts of *L. suffruticosum* without selection pressure (at 13 % final PEG-concentration.)

	experiment no.	number of protoplasts	number of calli	absolute plating efficiency	average
treatment for transformation	1	5.0×10^5	307	0.6×10^{-3}	1.0×10^{-3}
	2	5.0×10^5	620	1.2×10^{-3}	
	3	5.0×10^5	322	0.6×10^{-3}	
	4	5.0×10^5	771	1.5×10^{-3}	
control	1	4.0×10^5	4178	10.4×10^{-3}	9.7×10^{-3}
	2	4.0×10^5	3600	9.10×10^{-3}	

Plastocyte transformation in Linum usitatissimum

Plastocytes of six genotypes were used in transformation experiments with agrobacteria. Within 4–5 weeks after coculture with the bacteria, resistant calli of about 1 mm in diameter were formed (Fig. 3 a). Callus fragments showed a positive reaction in the histochemical assay of *GUS* gene activity. Resistant calli were observed in five of the six genotypes investigated (Table 3). On organogenesis medium, five callus lines of cv. Bionaa and one of cv. Langeland B regenerated shoots (Fig. 3 b). They showed high resistance against kanamycin sulfate and a positive reaction in the histochemical assay of *GUS*-activity. The shoots rooted on MS medium with 100 mg/L kanamycin sulfate.

In order to test the effect of the bacterium concentration, 5.0×10^5 protoplasts were cocultured after 3-days of preculture with 10^6 and 10^7 bacteria in 1 mL for 24 h. At the high concentration of bacteria, the average absolute transforma-

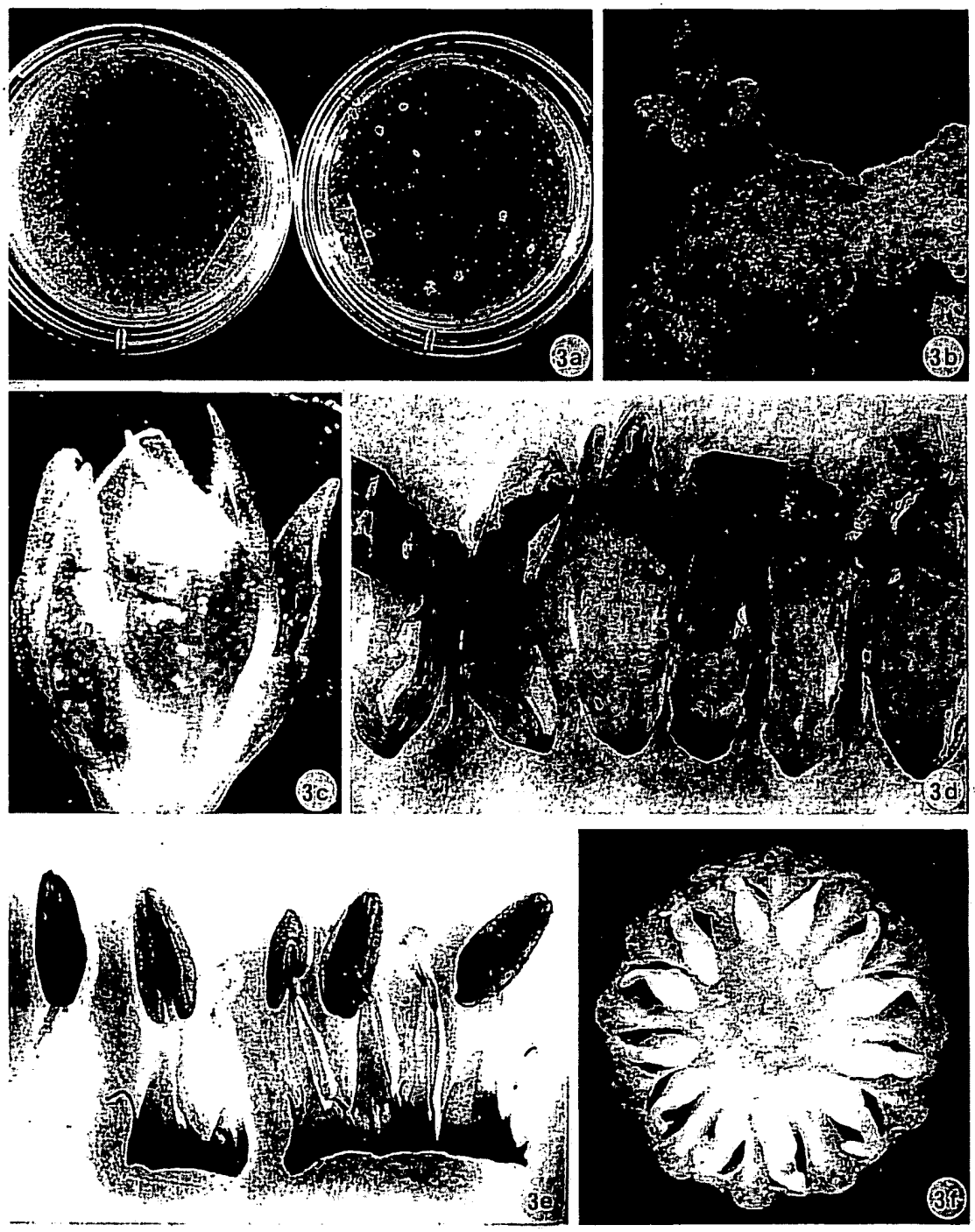

Fig. 3: Plastocyte transformation in *L. usitatissimum* mediated with the strain *Agrobacterium tumefaciens* GV2260 pBin19 35S*GUS*: 3 a) resistant calli (right) formed within 4 weeks in the selection medium V-KM containing 500 mg/L claforan and 50 mg/L kanamycin sulfate, and control (left); 3 b) adventitious shoot regeneration from a resistant callus of the genotype Bionaa on MS medium with 2.5 µmol/L BAP and 100 mg/L kanamycin sulfate; 3 c–f) increased number of flower organs in the transgenic lines TA6-47 in contrast to the pentamerous flowers of the wild type; 3 c) six sepals; 3 d) six petals of a flower; 3 e) seven stamina of a flower, two of them with rudimentary anthers; 3 f) cross section face of a capsule that had six carpels.

tion frequencies estimated by the number of resistant calli were twice as high as the low concentration in the genotypes Bionaa and C.A.Z. 2612-A (Fig. 4). With the genotypes Langeland B and Otofte 15/47, no significant data were obtained due to their low transformation rates. At the bacterium concentration of 10^7 the transformation frequencies appeared

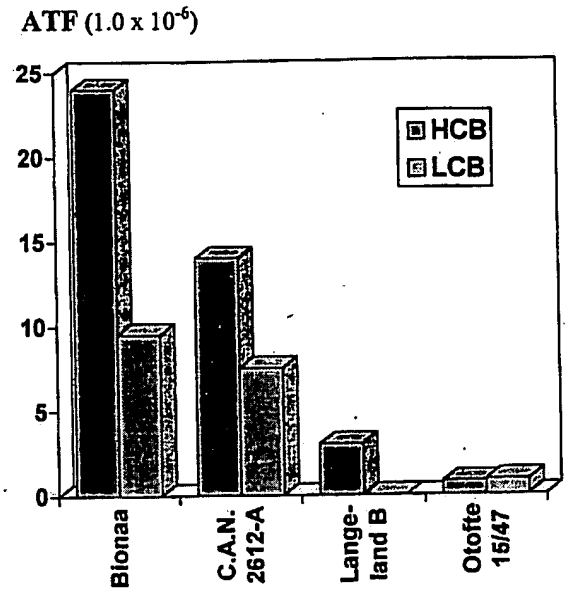

Fig. 4: Effects of *Agrobacterium* concentration on the absolute plastocyte transformation frequencies (ATF). An average of four experiments. HCB = 10^7 bacteria/ml plastocyte culture and LCB = 10^6 bacteria/mL plastocyte culture.

Table 2: Protoplast transformation frequencies in *L. suffruticosum*.

experiment	number of protoplasts	number of resistant calli	absolute transformation frequencies	relative transformation frequencies
1	0.9×10^6	3	3.3×10^{-6}	3.3×10^{-3}
2	0.5×10^6	1	2.0×10^{-6}	2.0×10^{-3}
3	0.6×10^6	1	1.7×10^{-6}	1.7×10^{-3}
4	1.5×10^6	3	2.0×10^{-6}	2.0×10^{-3}
5	1.5×10^6	3	2.0×10^{-6}	2.0×10^{-3}
sum	5.0×10^6	11	2.2×10^{-6}	2.2×10^{-3}

Table 3: Results of plastocyte transformation in *Linum usitatissimum*.

genotype	number of experiments	number of resistant calli	number of resistant calli with shoots
Bionaa	4	101	5
C.A.N.2612-A	4	57	0
Langeland B	4	30	1
Ottwa	4	28	0
Otofte 15/47	4	6	0
IR 00610	3	0	0

much divergent among the six genotypes investigated (Table 4). The cv. Bionaa revealed the highest transformation frequency. In the cv. IR 00610, no resistant callus was observed.

For the molecular identification of the genes introduced in cv. Bionaa, the total DNA of four lines was extracted from shoots. The DNA was digested with the restriction endonucleases EcoR I, Hind III and BamH I, respectively. Their

Table 4: Absolute plastocyte transformation frequencies in *Linum usitatissimum*.

genotype	number of protoplasts	number of resistant calli	absolute transformation frequencies
Bionaa	3.0×10^6	79	26.3×10^{-6}
C.A.N.2612-A	3.6×10^6	47	13.1×10^{-6}
Langeland B	3.5×10^6	19	5.4×10^{-6}
Ottawa	11.1×10^6	28	2.5×10^{-6}
Otofte 15/47	3.1×10^6	5	1.6×10^{-6}
IR 00610	2.4×10^6	0	0

Table 5: Plastocyte transformation frequencies in *Linum suffruticosum*.

experiment	number of protoplasts	number of resistant calli	absolute transformation frequencies
1	1.6×10^6	83	5.2×10^{-5}
2	1.1×10^6	38	3.3×10^{-5}
3	0.9×10^6	42	4.7×10^{-5}
sum	3.6×10^6	163	4.5×10^{-5}

blots were probed with DNA fragments of *NPT II*, *GUS*, and with the 35S promotor. All of the tested lines showed positive reactions with the three probes (Fig. 5). The different positions of the hybridization bands indicated that the T-DNA was inserted at different loci of the genomes. The copy numbers of the introduced genes were apparently one in line TA6–7 and two in the genomes of the other three lines. Slight cross hybridization to genomic DNA appeared with the sequences of *GUS* gene and the 35S promotor. The Southern blots prepared from resistant callus lines of genotype C.A.Z. 2612-A showed positive bands with the *NPT II* and *GUS* gene probes.

Plantlets of the four transgenic lines of the cv. Bionaa were transplanted in the growth chamber. They showed different morphological characters. The plants of TA6-41 and TA6-47 became larger than the wild type in all organs. About 60% of the flowers of lines TA6-41 and TA6-47 possessed 6–7 sepals, 6–8 petals, 6–7 anthers and 6 carpels in contrast to the usually pentamerous flowers (Figs. 3 c, 3 d, 3 e, 3 f). The leaves of the line TA6-10 were strongly recurved. The plants of line TA6-7 produced normal flowers. In line TA6-10, the flowers were smaller than the wild type, developed rudimental petals, often remained closed and frequently died off in early bud stages. The frutication was generally impaired in all of the four transgenic lines. In line TA6-47, many but mostly seedless capsules were developed. Only 10 seeds were found in the 97 mature capsules, whereas usually a single capsule of the wild type contains 10 seeds. In the line TA6-41, 3 seeds were obtained from 3 capsules. No fruit ripened in lines TA6-7 and TA6-10. The seeds of TA6-41 and TA6-47 were germinated on MS medium containing kanamycin. Five of the seven seedlings obtained in line TA6-47 possessed three cotyledons. The flower morphology was as abnormal as in the first generation. The seeds of the line TA6-41 did not germinate.

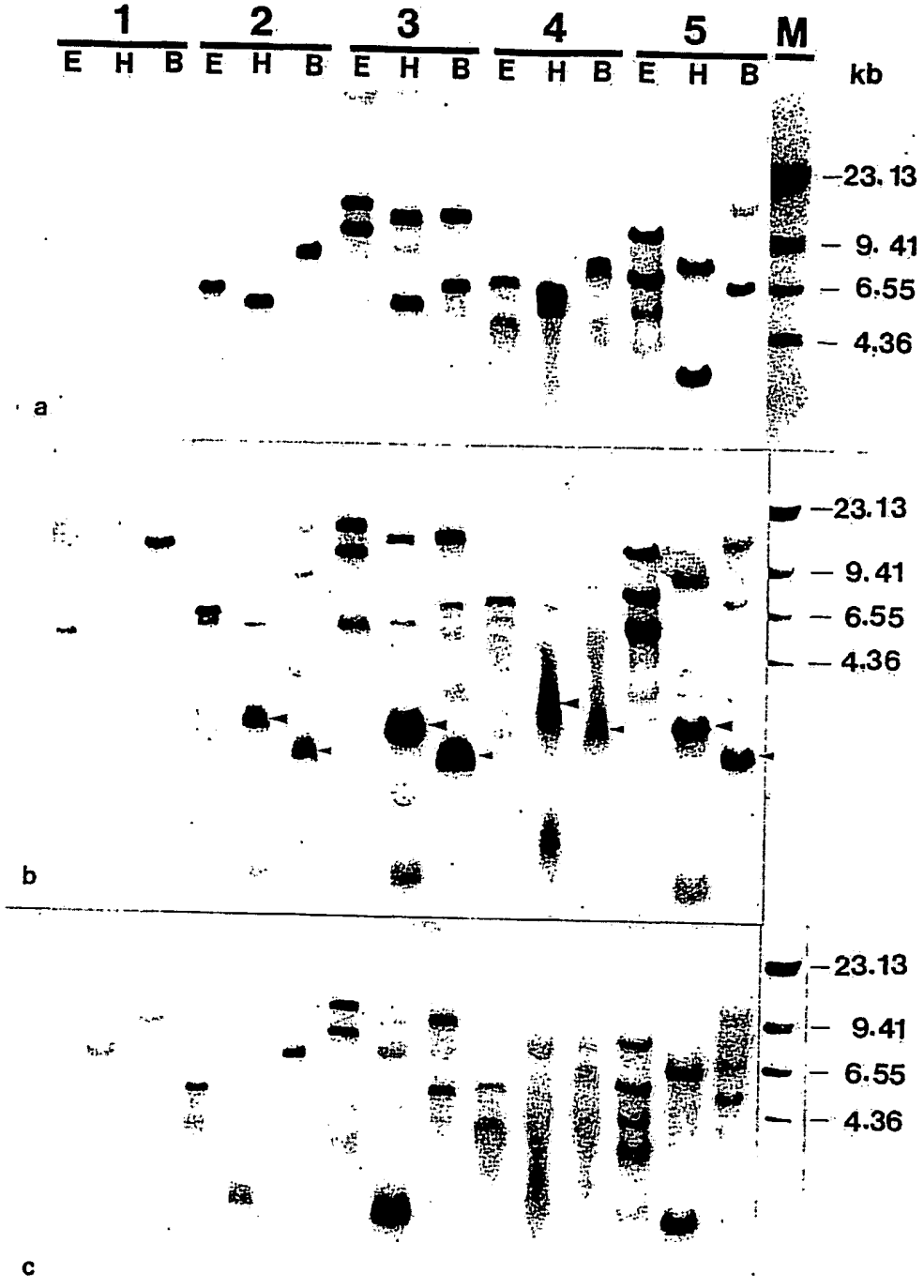

Fig. 5: Southern analysis of transgenic plants of the genotype Bionaa of *L. usitatissimum*. Total DNA was digested with EcoR I (E), Hind III (H) and BamH I (B), respectively, fractionated by agarose gel electrophoresis, transferred to a nylon membrane and probed with ^{32}P-labelled sequences of the *NPT II* gene (a), *GUS* gene (b) and 35S promotor (c), respectively; No. 1: wild type; Nos. 2–5: transgenic lines TA6-7, TA6-10, TA6-41 and TA6-47; M: molecular weight markers (λ-Hind III). In part b, the thick and thin arrow heads show separately the 2.5 kb and 2.0 kb of *GUS* gene fragments excised with Hind III (H) and BamH I (B) in Nos. 2–5. The positive signals in No. 1 (wild type) reveal cross hybridization of the *GUS* gene to genomic DNA. Slight cross hybridization to genomic DNA appeared also with the probe of the 35S promotor in part c (s. No. 1).

Plastocyte transformation in Linum suffruticosum

Transformation in plastocytes of *L. suffruticosum* was achieved with the *A. tumefaciens* strain C58C1 Rif® pGV3850*HPT*. At the time of transfer onto B5C medium with hygromycin B after 4 weeks of culture, the resistant calli had reached sizes of about 1–2 mm diameter. They regenerated shoots, which rooted on MS medium containing 5.0 µmol/L NAA.

From three experiments, 163 resistant callus lines were obtained. On the basis of these putative transformants, the absolute transformation frequency was 4.5×10^{-5} and the relative transformation rate based on the average plating efficieny of protoplasts (Table 1) amounted to 4.6×10^{-3} (Table 5).

The transformed shoots were highly resistant against hygromycin B, whereas wild type shoots died off within 10 days when planted on the selection medium. The total DNA of six transgenic lines was isolated and digested with the restriction enzymes BamH I and Hind III, respectively. Southern hybridization with the *HPT* gene probe showed that all lines contained the 1033 bp *HPT* sequence excised with BamH I and a single band at a different molecular weight by digestion with Hind III, whereas no hybridization signal appeared in the control. The singularity of bands with the Hind III indicated that one copy of the T-DNA from the vector pGV3850*HPT* was inserted because only one cut site of Hind III exists outside the *HPT* gene in the T-DNA.

Discussion

In these studies, transgenic plants of *Linum* species were firstly obtained through protoplast transformation with PEG and plastocyte transformation using *A. tumefaciens*. The stability of the transformation has been confirmed by the expression of the resistant phenotype in regenerants, by the integration of the transgene into high molecular weight DNA, as well as in *L. usitatissimum* by genetic transmission of the marker gene to the subsequent generation.

Protoplasts of *L. suffruticosum* were sensitive against PEG already at a concentration of 13 %. The tolerance of protoplasts against PEG also varied in other taxa. This is reflected by the optimum values given for transformation, being 28 % in *Nicotiana tabacum* and *N. plumbaginifolia* (Negrutiu et al., 1987), 20 % in *Arabidopsis thaliana* (Damm et al., 1989) and 13 % in *Brassica napus* and *B. nigra* (Köhler et al., 1989; Golz et al., 1990).

Molecular biological assays showed that all of the resistant lines of *L. suffruticosum* contained a *HPT*-gene sequence. In line Td18-2-1, the total *HPT* gene could not be excised with BamH I, while a small *HPT*-positive fragment appeared in the digestion with Hind III, although there is no cut position within the *HPT*-gene. This could have been due to a rearrangement of the marker gene during the transformation, to the insertion of a part of the *HPT*-gene closely to a Hind III cut site of the host DNA, or to a mutation in a HPT cut site. The function of the *HPT*-gene was apparently not injured, because the shoots of this line grew as well as the other lines on the selection medium. Bilang et al. (1991) reported that the activity of the *HPT*-gene was not reduced through shortening by 12 amino acids at the 3'-end. The length of the insertion fragment of the plasmid-DNA in plant chromosomes is generally random in the direct gene transformation (Potrykus et al., 1985).

In *L. usitatissimum*, the frequencies of plastocyte transformation varied with the genotypes. The differences are explained by different genetic preconditions for the transformation process and different regeneration capabilities of protoplasts. Different efficiences among various genotypes and species were also found and discussed with other species (Chabaud et al., 1988; Eapen et al., 1987; Gilissen et al., 1991; Komari, 1989; Negrutiu et al., 1987; Vries-Uijtwael et al., 1989). El-Kharbotly et al. (1995) indeed identified a genetic competence factor in potato.

The bacteria concentration was another parameter influencing the yields of transformants. An increased titre resulted in higher efficiences, a finding also reported for tobacco by Binns (1991). An additional genotype-dependence was visible with flax: The bacteria titre effect was more pronounced in the competent genotype Bionaa than in the less competent genotype Otofte 15/47.

The transgenic lines TA6-41 and TA6-47 showed abnormal flower morphologies, which were transmitted to the progeny. The variation could be due to a spontaneous mutation or a mutation by insertion of the T-DNA into a gene controlling the number of flower organs. The similarity of the respective phenotypes of two transformant lines, as well as the high degree of lines showing flower variation and sterility, are an unexplained phenomenon. Insertion mutations were described several times (Feldmann et al., 1989; Yoder, 1990; Van Lijsebettens et al., 1991).

Previous experiments on flax transformation have been performed with hypocotyl segments. In order to estimate the efficiencies of the three approaches (PEG/protoplasts, *Agrobacterium*/plastocytes, and *Agrobacterium*/hypocotyl), experiments have also been carried out with hypocotyls in the genotype C.A.N. 2612-A of *L. usitatissimum* (Ling, 1992). The genotype showed a relatively high transformation competence of plastocytes ($0.1–1.2 \times 10^{-2}$ of the relative transformation rates), but due to inefficient plant regeneration from the transgenic calli, the effectivity was much lower compared with the hypocotyl transformation (70 transgenic plant lines obtained from 150 inoculated hypocotyl tissues; Ling, 1992). In *L. suffruticosum*, *A. tumefaciens*-mediated plastocyte transformation was more effective (two-fold of relative transformation rate) than the PEG-mediated protoplast transformation. The comparison of the yields of transformants with the results obtained by other authors are less significant because of the different genotypes used. Anyhow, transformations with protoplasts and plastocytes are powerful alternatives for introduction of foreign genes in *Linum*. The transformation occurs at the 1 C or 2 C level of the protoplasts and plastocytes and the 1 C level of the division products, giving rise to uniform or ditypic calli. Hence, the chance of monotypic shoots is much higher than after the use of multicellular explants for transformation. With these, phenotype masking as described in transformation with tissues (McHughen and Jordan, 1989) does not occur often, making selection and gene expression analysis much less prone to artifactual results

(Draper et al., 1988). Furthermore, there is no need to use special DNA sequences for integration of DNA into plant genomes by direct gene transformation (Golz et al., 1990). Compared with transformation with hypocotyl in *Linum*, it took 3–6 weeks longer from protoplasts to transgenic plants. In conclusion, direct gene transfer into isolated protoplasts is the most suitable procedure with a genotype or species such as *L. suffruticosum*, which is easily regenerated, while with a species such as *L. usitatissimum*, which is recalcitrant to the protoplast technology, the transformation in a more efficiency-regenable tissue should be preferred (compare review, Potrykus, 1991). However, improvement of the protoplast regeneration system should be attempted with the recalcitrant species in order to make it optimum to the more convenient single-cell transformation systems.

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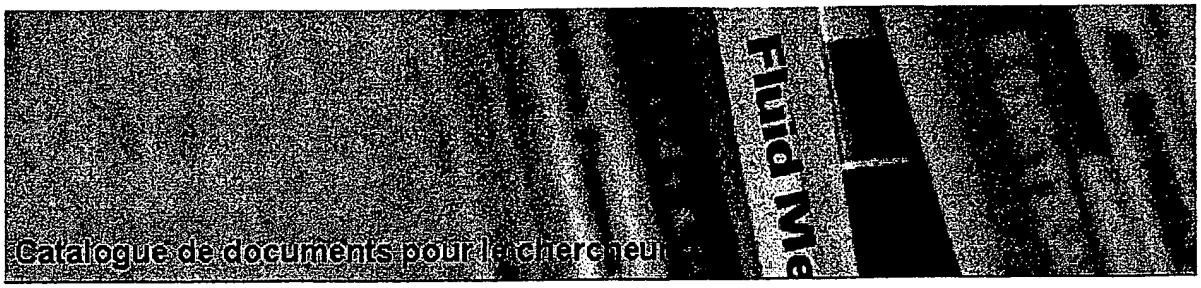

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Genetic transformation of Linum by particle bombardment

Auteur(s) / Author(s)
WIJAYANTO T. (1) ; MCHUGHEN A. (1) ;

Affiliation(s) du ou des auteurs / Author(s) Affiliation(s)
(1) Crop Development Centre, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK, S7N 5A8, CANADA
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Résumé / Abstract
Linseed flax (Linum usitatissimum L.) was transformed by bombarding hypocotyl tissues with gold particles coated with plasmid DNA carrying the β-glucuronidase (GUS) (uid-A) and neomycin phosphotransferase II (npt-II) genes. Transient expression of the introduced β-glucuronidase gene was used to study factors influencing the DNA delivery, while progeny analyses confirmed stable transformation. The efficiency of DNA delivery, uptake and expression was significantly affected by the duration of hypocotyl preculture, bombardment distances, the level of chamber vacuum, the quantity of DNA, and the size of particles. Nineteen independent GUS-positive shoots were recovered and regenerated into whole plants, from which 10 plants successfully produced viable seeds. Analysis of T[1] and T[2] self pollinated progeny for histochemical and fluorometric GUS assays and polymerase chain reaction (PCR) analyses for uid-A, plus npt-II PCR and germination assays in progeny plants demonstrated that the transgenes were expressed in selected plants and transmitted to progeny, usually via a single Mendelian locus. The results show that particle bombardment can be used to produce transgenic Linum plants. The system is rapid, simple and offers an alternative to Agrobacterium methods.

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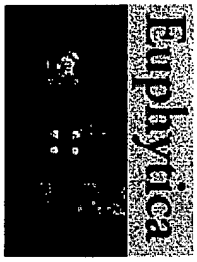

Herbicide resistant transgenic flax field test: Agronomic performance in normal and sulfonylurea-containing soils

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Alan McHughen[1] and Frederick Holm[1]

(1) Crop Development Centre, Dept. of Crop Science and Plant Ecology, University of Saskatchewan, S7N OWO Saskatoon, Sask., Canada

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Summary Two linseed flax (*Linum usitatissimum*) lines transformed with a mutant Arabidopsis ALS gene conferring resistance to sulfonylurea herbicides were tested in a replicated, randomized field test against its nontransformed commercial cultivar parent (cv. NorLin) in normal soil and in soil containing the commonly used sulfonylurea herbicides chlorsulfuron (Glean®) or metsulfuron methyl (Ally®). There were no significant differences between the transgenic lines and the parent

Referenced by

7 newer articles

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for any agronomic trait measured in untreated soil, indicating that there is no detrimental effect of T-DNA or foreign gene expression. Similarly, there were no significant differences for performance of the transgenic lines between the untreated and the herbicide treated soils, indicating that the transferred gene does confer a field level of tolerance to the flax. The control NorLin was devastated by the presence of the herbicides in the soil.

Key words field test - flax - herbicide resistan - linseed - Linum usitatissimum - transgenic

Abbreviation *ALS* acetolactate synthase

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